

In Vitro Evaluation of Nebulization Properties, Antimicrobial Activity, and Regional Airway Surface Liquid Concentration of Liposomal Polymyxin B Sulfate

Tejas R. Desai,¹ Gregory J. Tyrrell,² Tina Ng,² and Warren H. Finlay^{1,3}

Received October 25, 2002; accepted November 5, 2002

Purpose. To manipulate the activity of polymyxin B sulfate (PXB sulfate) by encapsulation in liposomes derived from appropriately selected surfactants that exhibit optimum entrapment and aerosol delivery of encapsulated PXB sulfate.

Methods. A combination of phospholipid (DMPG) and nonionic surfactants (Span 20 + Tween 80) was selected to encapsulate PXB sulfate. The nebulization properties were evaluated by nebulizing the liposomal dispersions with Pari LC Star nebulizers. The *in vitro* antibacterial activities of the original and nebulized liposomal formulations were evaluated against *Pseudomonas aeruginosa* (ATCC 27853) strains by broth microdilution, and their minimum inhibitory concentrations (MICs) were compared with those of free PXB sulfate and colistin methanesulfonate. Measurements of the aerosol properties during nebulization were used as input for a mathematical model of airway surface liquid in the lung of an average adult, to estimate the airway surface liquid concentration of the deposited liposomal PXB sulfate.

Results. The selected combination of surfactants showed maximum nebulization efficiency without compromising liposomal integrity during nebulization. PXB sulfate was added at a concentration of 10 mg/ml, and a molar ratio of PXB sulfate to dimyristoyl phosphatidylglycerol (DMPG) (sodium salt) of 1:5 was required to achieve 100% entrapment of PXB sulfate and no leakage on nebulization. Another formulation comprising half the concentrations of the optimized nonionic surfactants and DMPG was prepared to achieve a balance between the toxicity and efficacy after nebulization of encapsulated PXB sulfate. The *in vitro* antibacterial activities against *Pseudomonas aeruginosa* indicated that the activity of PXB sulfate could be manipulated by appropriate concentrations of the selected surfactants to achieve activity equivalent to that of colistin methanesulfonate, which is known to be less toxic than unencapsulated PXB sulfate. The estimated airway surface liquid concentrations of the deposited liposomal PXB sulfate reveal that the MIC of the nebulized liposomal PXB sulfate can be achieved over most of the tracheobronchial region, using a jet nebulizer with a volume fill of 2.5 ml or more.

Conclusions. It was established from this study that the encapsulation of PXB sulfate in liposomes reduces its activity against *P. aeruginosa* strains. Concentrations of PXB sulfate deposited in the tracheobronchial

region, predicted using a mathematical model, were above the measured MICs except in the case of very high mucus production rate and low mucus velocities.

KEY WORDS: polymyxin B sulfate; liposomes; aerosols; nebulization efficiency; lung deposition simulation.

INTRODUCTION

Pseudomonas aeruginosa is a predominant respiratory pathogen that plays an important role in the progressive lung destruction and subsequent respiratory failure that occurs in patients with cystic fibrosis (CF) (1,2). *P. aeruginosa* infections of the lower respiratory tract can range in severity from colonization to necrotizing bronchopneumonia. It is almost impossible to eradicate these Gram-negative bacteria once they become established in the airways. However, before this stage, aggressive treatment with suitable antibiotics can delay the development of chronic infection (3).

Polycationic peptides are a potent class of antibacterial agents that are bactericidal to Gram-negative bacteria (such as *P. aeruginosa*) (4,5). The mode of action comprises two major steps, binding to and permeabilization of the outer membrane, and induction of lethal leakages of the cytoplasmic components. Polymyxin B (PXB) sulfate and colistin are commonly used polypeptides known to be effective against *P. aeruginosa* (6). The above polypeptides are currently enjoying a resurgence, with use by inhalation and intravenous routes in the treatment of chronic infections in CF patients. However, *in vivo* experimental data have shown that these antimicrobials are rapidly cleared from the lungs of several animal models, resulting in less time of exposure to the bacteria (7). In addition, these antimicrobials are reported to cause severe toxic side effects (8). The latter problem in the case of colistin was addressed by synthesizing its methanesulfonate derivative, which is reported to be less toxic (6). PXB is currently not available in its methanesulfonate form, and hence, it is seldom used.

Encapsulation in colloidal carriers such as liposomes has been suggested as a way to reduce the pulmonary clearance of PXB sulfate (9). As encapsulation of drugs in liposomes is also known to reduce toxic side effects and alter the pharmacokinetics (10,11), it is reasonable to propose that liposomal PXB sulfate will be advantageous with reduced toxicity and extended pulmonary residence time. McAllister *et al.* verified this assumption by investigating the antimicrobial efficacy of liposomal PXB sulfate using cell-kill and MIC determinations against *P. aeruginosa* strains (12).

Aerosol technology for pulmonary drug delivery is a potential route for the delivery of therapeutic agents for the treatment of pulmonary diseases (13,14). The advantage of the aerosol mode of delivery is that the drug is deposited more uniformly over the respiratory tract, leading to local levels of the drug that may exceed the levels achieved by systemic administration. Colistin and aminoglycosides (such as tobramycin and gentamicin) are currently the most commonly prescribed antibacterial agents by the nebulization route. Beaulac *et al.* investigated the aerosolization of liposomal tobramycin in an animal model of chronic pulmonary infection caused by *P. aeruginosa* (15). No attempts have been made to investigate the nebulization behavior of the

¹ Aerosol Research Laboratory of Alberta, Department of Mechanical Engineering, University of Alberta, Edmonton, Alberta T6G 2G8, Canada.

² The Provincial Laboratory for Public Health-Alberta and the Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, Alberta, T6G 2G8, Canada.

³ To whom correspondence should be addressed. (e-mail: warren.finlay@ualberta.ca)

above mentioned polypeptides encapsulated in liposomes. Indeed, this is what we have begun to investigate. The objective of the present study was to achieve a nebulizable liposomal formulation of PXB sulfate and to find a way to manipulate the activity of PXB sulfate using its electrostatic interactions with charged phospholipids in a way that the liposomal PXB sulfate shows less or equivalent toxicity in comparison with colistin methanesulfonate. Antimicrobial studies were performed by measuring and comparing the activities of liposomal PXB sulfate and nonentrapped (free) PXB sulfate against *P. aeruginosa* strains and compared with the activity of colistin methanesulfonate. A mathematical model of airway surface liquid (ASL) was used to estimate the local PXB sulfate concentrations in each generation of the lung after aerosolization.

MATERIALS AND METHODS

Materials

Dimyristoyl phosphatidylglycerol (DMPG) (sodium salt) (>99%) was purchased from Genzyme Pharmaceuticals (Cambridge, MA, USA). Egg phosphatidylcholine (EPC) (>99%) was purchased from Avanti Polar Lipids (Alabaster, Alabama). Tween 80 (Polysorbate 80) and Span 20 (sorbitan monolaurate) were purchased from Aldrich Chemical Company (Milwaukee, Wisconsin). Cholesterol, solid PXB sulfate (USP compatible, 7740 U/mg), colistin sulfate salt (USP compatible, 20280 U/mg), and ninhydrin were purchased from Sigma-Aldrich, Canada. For thin layer chromatography (TLC), silica gel plates on aluminum foil were purchased from Whatman Ltd., Kent, UK. The *P. aeruginosa* strain ATCC 27853 was used in this study. Organic solvents such as chloroform, ammonium hydroxide, 1-butanol, and methanol were purchased from Fisher Scientific, Canada. All the solvents were analytic or HPLC grade. All the materials were used as received.

Methods

Preparation of Liposomes

Liposomal dispersions were prepared by a thin-film hydration technique. Phospholipid with or without nonionic surfactants was dissolved in a mixture of chloroform and methanol in a round-bottom flask. The solvent was evaporated using a rotary evaporator to form a thin film. Traces of solvents were removed by drying the film in a vacuum oven for 2 h at <40°C. The film was then hydrated with PXB sulfate solution in 0.9% saline. PXB sulfate was added at a concentration of 10 mg/ml. The dispersion was then equilibrated at room temperature ($24 \pm 1^\circ\text{C}$) for an hour. The entrapment of PXB sulfate in liposomes was determined by centrifuging the dispersion at $21,460 \times g$ and 4°C for 90 min and testing the supernatant and pellet for the presence of PXB sulfate by TLC. Developing solvent for TLC was prepared by mixing methanol, 1-butanol, aqueous ammonia, and chloroform in the ratio of 14:4:9:12 by volume, as described by Krzek *et al.* (16). The spot was developed using 0.2% ninhydrin solution in methanol as a visualizing agent. If the spot was observed, then the concentration of PXB sulfate was quantified using UV spectrophotometry (Hewlett Packard 8452A) ($\lambda_{\text{max}} = 254 \text{ nm}$).

Nebulization of Liposomal PXB Sulfate

A volume fill of 2.5 ml of liposomal dispersion was nebulized with Pari LC STAR (Pari, Starnberg, Germany) jet nebulizers using a DeVilbiss Pulmo Aide Compressor (Model 5650D). Three different nebulizer units were used in this study. The Pari LC STAR nebulizer was selected for this purpose because it is reported to have shown superior performance in previous studies (17). The aerosol was collected on Respigard filters (Marquest Medical Products, Englewood, Colorado). Usually two or three filters are connected to each other in series. After nebulization, the filter contents were extracted with 0.9% saline to determine the nebulization efficiency. Nebulization efficiency of a liposomal formulation is defined as the total output of drug (PXB sulfate) collected on the filters from the nebulizer calculated as a percentage of the total submitted to nebulization. In other words, after assaying PXB sulfate content extracted from the filters, nebulization efficiency may be determined as:

$$\text{Nebulization efficiency (\%)} = 100 \times$$

$$[\text{aerosolized PXB (collected on filters)}]/(\text{total PXB sulfate submitted to nebulization})$$

To determine the leakage during nebulization, a portion of the sample was centrifuged at $21,460 \times g$ and 4°C for 90 min. The supernatant was assayed for free PXB sulfate, and the pellet was dissolved in methanol and assayed for encapsulated PXB sulfate by UV spectrophotometry. The percentage of the drug encapsulated was calculated as the ratio of the drug in the pellet to the sum of the drug in the pellet and drug in the supernatant. Samples of the original residual preparations remaining in the nebulizer after nebulization were also submitted to the same procedure.

Broth Microdilution Susceptibility Assay

Minimum inhibitory concentrations (MICs) of free, liposomal, and nebulized liposomal PXB sulfate were determined by broth microdilution in Mueller-Hinton broth according to NCCLS standards (18). All the preparations assayed were tested against the strain of *P. aeruginosa* ATCC 27853. *P. aeruginosa* was grown for 20 to 24 h on sheep blood agar. Inocula were prepared by suspending the bacteria in sterile saline to obtain a turbidity equivalent to that of a McFarland standard of 0.5. The suspension was further diluted to provide a final inoculum concentration of approximately $5 \times 10^5 \text{ CFU}/\text{ml}$ in the wells of the broth microdilution trays. The microdilution trays were incubated at 35°C for 20 to 24 h before determination of MICs.

Particle Size and Zeta Potential Measurement of Liposomal PXB Sulfate

To investigate the effect of drug loading on liposome surface charge, zeta potentials of the empty and loaded vesicles were measured. The particle size and zeta potential measurements were performed using Zetasizer 3000HS (Malvern Instruments Ltd., Southborough, Massachusetts) at $25 \pm 0.1^\circ\text{C}$.

Particle Size Measurement of Aerosolized Liposomal PXB Sulfate

Size distribution of the aerosolized particles was determined using a phase Doppler anemometer (PDA) (Dantec

Electronics Inc., Mahwah, New Jersey), as described by Prokopp *et al.* (19).

Numerical Predictions

The efficacy of a drug intended for local antimicrobial activity in the human airways depends in part on its local concentration in the mucus. For this reason, a mathematical model previously described by us (25) was used to estimate the airway surface liquid concentrations of PXB sulfate. Briefly, the volume of periciliary liquid (PCL) in each lung generation was calculated using cilia length (20) and the morphometric dimension from the respective lung model. The thickness of the mucus layer was estimated using mass conservation and models of average mucus velocity and production rate in each lung generation. The mucus velocities were calculated by considering the *in vivo* data of the mucociliary clearance rates, as explained in detail by Finlay *et al.* (25). The lung of a healthy adult was modeled as a symmetrically branching model (17) derived from literature data for the conducting airways (generations 0 to 14) given by Phillips *et al.* (21) and for the alveolar region by Haefeli-Blueuer and Wiebel, scaled to a 3-L lung volume (22).

Because of variability in the mucus production rates and velocities, two combinations of each were chosen that would result in lower and upper limits of the estimated PXB sulfate concentration in the ASL. A maximum tracheal velocity of 15 mm/min was combined with an arbitrarily low daily mucus production rate (5 ml/day) to estimate the maximum PXB sulfate concentrations in the ASL (23). A high mucus production rate of 40 ml/day (24) together with a relatively low tracheal mucus velocity of 5 mm/min was selected as the extreme, to predict the minimum concentration levels of liposomal PBX sulfate in the ASL. It should be noted that the values of the concentration of liposomal PXB sulfate in the total ASL were estimated by assuming uniform deposition of liposomal PXB sulfate in each generation, followed by homogeneous dispersion in the ASL. The resulting estimates of the concentration of liposomal PXB sulfate represent the values in the state that exists after nebulization and before significant mucociliary clearance. The concentration of the liposomal PXB sulfate deposited in each generation of the lung estimated using this model was compared with the MIC concentrations to evaluate delivery of the formulations described in this paper.

RESULTS AND DISCUSSION

Various liposomal formulations were prepared and compared in terms of the encapsulation efficiency, leakage on nebulization, and nebulization efficiency. The liposomal preparation containing EPC and cholesterol (molar ratio of 1:1) encapsulating PXB sulfate, prepared by film hydration technique, formed large agglomerates during nebulization, thereby showing extremely low nebulization efficiency. Similar problems associated with nebulization of liposomal formulations containing cholesterol were observed previously by other researchers (25). In our recent study, we reported the feasibility of encapsulating drugs in spontaneously formed liposomes resulting by mere dispersion of micronized mixtures of phospholipid(s) and drugs in the aqueous environment (26). Liposomal PXB sulfate dispersion containing DMPG

(concentration 35 mM) was prepared in a similar manner, and the nebulization properties of the spontaneously formed liposomes encapsulating PXB sulfate were investigated. DMPG was selected for this purpose because PXB sulfate is cationic in nature, and it was observed in our previous study that incorporation of a cationic compound in spontaneously formed negatively charged liposomes would increase its uptake (26). The preparation showed excellent entrapment efficiency (100%) of PXB sulfate in spontaneously formed liposomes. However, the delivery on nebulization of PXB sulfate encapsulated in liposomes was very poor.

In an attempt to achieve a nebulizable formulation, non-ionic surfactants such as Tween 80 and Span 20 were incorporated in DMPG. After optimization of the various parameters, Formulation 1 shown in Table I was obtained, which showed excellent encapsulation and nebulization properties. The average particle size of the liposomal PXB sulfate dispersion, as determined by photon correlation spectroscopy and peak analysis by volume, was 427.3 nm with 85% of the liposomes in the range 349–553 nm, 12% in the range 110–277 nm, and 3% in the range 697–877 nm. The zeta potential of the liposomal PXB sulfate preparation was found to be -6.9 ± 2.3 mV. Nebulization properties of the formulation were evaluated in terms of nebulization efficiency, encapsulation efficiency of PXB sulfate in nebulized liposomes collected on the filter, and encapsulation efficiency in the residual liposomal preparation left in the reservoir after nebulization. The results are shown in Table II. The mass median aerodynamic diameter (MMAD) value for nebulized Formulation 1 was found to be 4.65 ± 0.05 μm , and the geometric standard deviation (GSD) was 1.52 ± 0.01 .

It is known that complex formation between the phospholipid and the drug is one of the mechanisms of encapsulation of drug (27). Because 100% encapsulation of PXB sulfate was obtained in our formulation, before and after nebulization, it may be feasible to assume that the negatively charged phospholipid (i.e., DMPG) was able to block all the five primary amino groups present in PXB sulfate by complex formation. It should also be noted that the molar ratio of PXB sulfate and the phospholipid (i.e., DMPG) in Formulation 1 is 1:5.

The objective of the study was to achieve a balance between the toxicity and the efficacy of PXB sulfate, and so a formulation comprising half the concentrations of all the excipients was prepared and described as Formulation 2 in Table I. The rationale behind preparing this formulation was to block two or three (or fewer than five) primary amino groups of PXB sulfate and to understand its impact on nebulization properties and the MIC of nebulized liposomal PXB sulfate against *P. aeruginosa*. The concentration of PXB sulfate was kept constant (i.e. 10 mg/ml). The nebulization efficiency, encapsulation efficiency of PXB sulfate in nebulized liposomes collected on the filter, and encapsulation efficiency

Table I. Formulations Used for Nebulization of Liposomal PXB

Component	Formulation 1 (mg/ml)	Formulation 2 (mg/ml)
DMPG	23.8	11.9
Tween 80	3.50	1.75
Span 20	4.14	2.07
PXB	10	10

Table II. Comparison of Various Parameters following Nebulization of Liposomal Formulations 1 and 2 (Mean \pm SD, n = 3):

Parameter	Formulation 1	Formulation 2
Original encapsulation (%)	100	44.8 \pm 5.6
Nebulization efficiency (%)	52.3 \pm 6.6	55.4 \pm 2.8
Entrapment of PXB in nebulized liposomes (%)	100	26.8 \pm 2.3
Entrapment of PXB in residual dispersion in nebulizer (%)	100	41.0 \pm 4.7

of PXB sulfate in the residual preparation left in the reservoir after nebulization for Formulation 2 are shown in Table II. As can be seen, reducing the concentrations of DMPG, Tween 80, and Span 20 by half reduced the encapsulation efficiency to 44.8 \pm 5.6%. Similarly lower encapsulation was observed in the nebulized preparation extracted from the filter (26.8 \pm 2.3%) and the residual dispersion in the nebulizer (41.0 \pm 4.7%). These results show that the delivery of encapsulated PXB sulfate after nebulization is lower than that in Formulation 1. The average particle size of liposomes in Formulation 2, as determined by photon correlation spectroscopy and peak analysis by volume, was found to be 396 nm with 70% of the liposomes between 418 and 636 nm and 30% in the range 120–207 nm. The zeta potential of this liposomal PXB sulfate preparation was found to be -0.4 ± 2.1 mV. It can be seen that the negativity of the zeta potential value for Formulation 2 is lower, indicating less blockage of the amino groups compared to Formulation 1.

Antimicrobial Activity

The MICs of free, liposomal, and nebulized liposomal PXB sulfate for Formulations 1 and 2 against *P. aeruginosa* ATCC 27853 are shown in Table III. The MIC of colistin methanesulfonate against *P. aeruginosa* is also reported in Table III for comparison purposes, as colistin methanesulfonate is reported to be less toxic than PXB sulfate (6). As can be seen, the MIC of free PXB sulfate is less than that of free colistin methanesulfonate, thereby confirming the lower activity of colistin methanesulfonate. Empty liposomal preparations showed no activity against the strains of *P. aeruginosa*, thereby indicating that there was no interaction between the excipients and bacteria. It can be seen from Table III that the MIC of liposomal PXB sulfate (Formulations 1 and 2) was higher than that of free PXB sulfate, thereby indicating that the *in vitro* activity of PXB sulfate was reduced by encapsulation in liposomes. A similar observation was previously reported for the aminoglycoside amikacin, encapsulated in anionic liposomes by Omri *et al.* (28). The higher activity of Formulation 2 compared to Formulation 1 may be attributed to lower encapsulation in the former and availability of a higher fraction of free PXB sulfate compared to the Formulation 1. It may be noted that the MIC of Formulation 1 is increased after nebulization. The process of nebulization is reported to increase the MIC by one to two dilutions, which, however, is considered negligible (29). It is also interesting to note that the liposomal PXB sulfate shows either the same

activity as (Formulation 2) or lower (Formulation 1) activity than colistin methanesulfonate.

Numerical Predictions

From the experimental data on the particle size distribution of aerosolized droplets and the total output after nebulization, the deposited dosages of liposomal PXB sulfate in each lung generation were estimated using the mathematical model for a given volume (2.5 ml) of nebulized Formulation 1. The estimated bounds of the liposomal PXB sulfate concentration in the ASL along the tracheobronchial airways of an adult for Formulation 1 are shown in Fig. 1. It can be seen from Fig. 1 that the estimated concentration of liposomal PXB sulfate is higher in the proximal airways, gradually reducing in the more distal bronchioles. For a low mucus production rate of 5 ml/day, the deposited concentrations of PXB sulfate predicted by this model, in generations 0 to 7, were above the MIC of the nebulized formulation (62.5 μ g/ml) for both extremes of tracheal mucus velocities (5 mm/min and 15 mm/min). However, the estimated concentration of PXB sulfate in generations 8 to 14 was below the MIC of 62.5 μ g/ml. For a high mucus production rate of 40 ml/day with a high tracheal mucus velocity of 15 mm/min, the estimated concentration of the aerosolized PXB sulfate was above the MIC only in generations 0 to 5. In the case of a low tracheal mucus velocity of 5 mm/min with a high mucus production rate of 40 ml/day, the estimated concentration of PXB was found to be below the MIC in all the generations. It should, however, be noted that this is an extreme case and rarely occurs in adults. It should also be noted that the predicted dosages of deposition of PXB sulfate at the given mucus production rates and tracheal mucus velocities correspond to the nebulization of a volume of 2.5 ml of Formulation 1. Concentrations of PXB sulfate lower than the MIC might be overcome by nebulizing a higher volume of the liposomal PXB, as it is known that the nebulization of higher volumes results in greater deposition of drug (30). Nevertheless, these simulation results combined with the antimicrobial results indicate that the delivery of optimized liposomal PXB sulfate via aerosol route is a viable delivery option to reduce the toxicity of PXB sulfate, thus

Table III. *In Vitro* Activities of Free, Liposomal, and Nebulized Liposomal PXB and Free Colistin Methanesulfonate against *P. Aeruginosa* (ATCC 27853) Strain

Component	MIC before nebulization (μ g/ml)	MIC after nebulization (μ g/ml)
Free PXB sulfate	3.9–7.8	NA
Free colistin methanesulfonate	15.6	NA
Liposomal PXB sulfate (Formulation 1)	31.3	62.5
Liposomal PXB sulfate (Formulation 2)	7.8–15.6	15.6
Empty liposomes (Formulation 1)	>1000	NA
Empty liposomes (Formulation 2)	>1000	NA

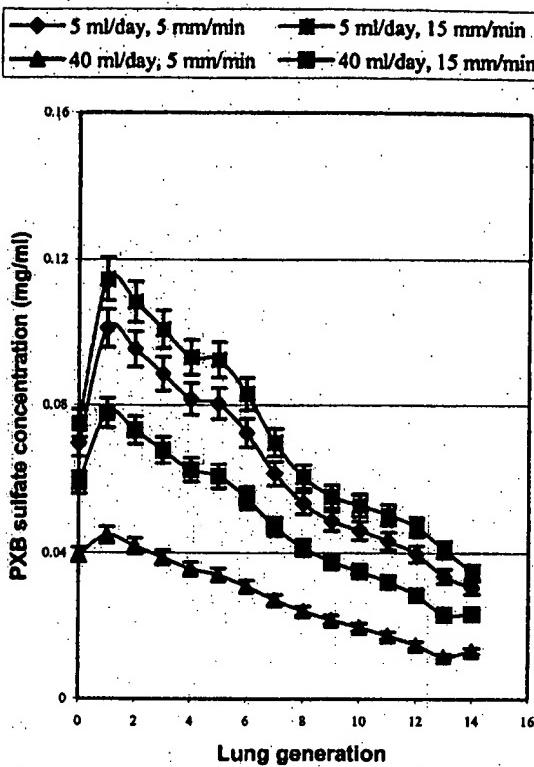


Fig. 1. Predicted generational concentrations of liposomal PXB sulfate in the ASL after nebulization for an average adult, at different mucus production rates and tracheal mucus velocities ($n = 3$, mean \pm SD).

fulfilling the main objective of the present work. This finding may open the door for future *in vivo* testing of these developed liposomal PXB sulfate formulations and may accelerate the consideration of PXB sulfate as a potent antimicrobial agent that is as effective and efficacious as colistin methanesulfonate.

CONCLUSIONS

In this paper we have successfully demonstrated the feasibility of delivering liposomal PXB sulfate by nebulization. We have also demonstrated a way to manipulate the activity of liposomal PXB sulfate dispersions by changing the concentration of surfactants to block fewer amino groups in PXB sulfate. The change in the antibacterial activity was investigated for these liposomal dispersions and the nebulized liposomal aerosols generated from these dispersions. The *in vitro* antibacterial activity was compared against that of free colistin methanesulfonate, which is known to have lower toxicity than PXB sulfate. It was established from this study that the encapsulation of PXB sulfate reduces its activity against *P. aeruginosa* strains, with activity equal to or less than that of colistin methanesulfonate. A mathematical model was used to estimate the regional deposition and airway surface liquid concentration of liposomal PXB sulfate in various generations of the lung. Concentrations above the measured MICs were predicted in the tracheobronchial region, except in the case of very high mucus production rate and low mucus velocities. These data lend a voice in favor of polymyxins for potential use in the treatment of respiratory diseases and may open the door for future *in vivo* testing.

ACKNOWLEDGMENTS

The authors also wish to thank Helena Orszanska for excellent laboratory help and cooperation during this work. W.F. gratefully acknowledges the support of the Canadian Cystic Fibrosis Foundation's SPARx program in this work.

REFERENCES:

- T. B. May, D. Shinabarger, R. Maharaj, J. Kato, L. Chu, and J. D. DeVault. Alginate synthesis by *Pseudomonas aeruginosa*: a key pathogenic factor in chronic pulmonary infections of cystic fibrosis patient. *Clin. Microbiol. Rev.* 4:191–206 (1991).
- B. van Klingerden. Antibiotic resistance in *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Staphylococcus aureus*. *Chest* 94:S103–S109 (1988).
- D. J. Touw, R. W. Brimicombe, M. E. Hodson, H. G. M. Heijerman, and W. Bakker. Inhalation of antibiotics in cystic fibrosis. *Eur. Respir. J.* 8:1594–1604 (1995).
- D. R. Storm, K. S. Rosenthal, and P. E. Swanson. Polymyxin and related peptide antibiotics. *Ann. Rev. Biochem.* 46:723–763 (1977).
- T. Jensen, S. S. Pedersen, S. Garne, C. Heilmann, N. Hoiby, and C. Koch. Colistin inhalation therapy in cystic fibrosis patients with chronic *Pseudomonas aeruginosa* lung infection. *J. Antimicrob. Chemother.* 19:831–838 (1987).
- M. E. Evans, D. J. Feola, and R. P. Rapp. Polymyxin B sulfate and colistin: Old antibiotics for emerging multiresistant gram negative bacteria. *Ann. Pharmacother.* 33:960–967 (1999).
- H. Schreier, R. J. Gonzalez-Rothi, and Á. A. Stecenko. Pulmonary delivery of liposomes. *J. Control. Release* 24:209–223 (1993).
- W. A. Craig and C. M. Kunin. Dynamics of binding and release of the polymyxin antibiotics by tissues. *J. Pharmacol. Exp. Ther.* 184:757–765 (1973).
- S. M. McAllister, H. O. Alper, Z. Teitelbaum, and D. B. Benett. Do interactions with phospholipids contribute to the prolonged retention of polypeptides within the lung? *Adv. Drug Deliv. Rev.* 19:89–110 (1996).
- J. N. Weinstein and L. D. Leserman. Liposomes as drug carriers in cancer chemotherapy. *Pharmacol. Ther.* 24:207–233 (1984).
- G. Gregoridis. *Liposomes as Drug Carriers*. J. Wiley & Sons, London, 1988.
- S. M. McAllister, H. O. Alper, and M. R. W. Brown. Antimicrobial properties of liposomal polymyxin B. *J. Antimicrob. Chemother.* 43:203–210 (1999).
- F. Morén and S. P. Newman. Aerosol dosage forms and formulations. In: F. Morén, M. B. Dolovich, and M. T. Newhouse (Eds.), *Aerosols in Medicines*. Elsevier, New York, 1993, p.321.
- W. H. Finlay. *The Mechanics of Inhaled Pharmaceutical Aerosols: An Introduction*. Academic Press, Oxford, 2001.
- C. Beaulac, S. Sachetelli, and J. Lagace. Aerosolization of low phase transition temperature liposomal tobramycin as a dry powder in an animal model of chronic pulmonary infection caused by *Pseudomonas aeruginosa*. *J. Drug Target.* 7:33–41 (1999).
- J. Krzek, M. Starek, A. Kwiecien, and W. Rzeszutko. Simultaneous identification and quantitative determination of neomycin sulfate, polymyxin B sulfate, zinc bacitracin and methyl and propyl hydroxybenzoates in ophthalmic ointment by TLC. *J. Pharm. Biomed. Anal.* 24:629–636 (2001).
- W. H. Finlay, C. F. Lange, M. King, and D. P. Spreet. Lung delivery of aerosolized dextran. *Am. J. Respir. Crit. Care Med.* 161:91–97 (2000).
- National Committee for Clinical Laboratory Standards. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, 2nd ed.: Approved Standard M7-A2, NCCLS, Villanova, Pennsylvania, 1990.
- R. M. Prokop, W. H. Finlay, and K. W. Stapleton. An *in vitro* technique for calculating the regional dosages of drugs delivered by an ultrasonic nebulizer. *J. Aerosol Sci.* 26:847–860 (1995).
- J. G. Widdicombe. Airways surface liquid: Concepts and measurements. In: D. F. Rogers and M. I. Lethem (eds). *Airway Mucus: Basic Mechanism and Clinical Perspectives*, Birkhauser, Basel, 1997, pp. 1–17.
- C. G. Phillips, S. R. Kaye, and R. C. Schroter. A diameter-based

- reconstruction of the breathing pattern of the human bronchial tree. Part I. Description and application. *Respir. Physiol.* 98:193-217 (1994).
- 22. B. Haefeli-Bleuer and E. R. Weibel. Morphometry of the human pulmonary acinus. *Anat. Rec.* 220:401-414 (1988).
 - 23. D. B. Yeates, J. M. Sturgess, S. R. Kahi, H. Levison, and N. Aspin. Mucociliary transport in trachea of patients with cystic fibrosis. *Arch. Dis. Childhood* 51:28-33 (1975).
 - 24. B. Oberwaldner, J. C. Evans, and M. S. Zach. Forced expirations against a variable resistance: A new chest physiotherapy method in cystic fibrosis. *Pediatr. Pulmonol.* 2:358-367 (1986).
 - 25. C. F. Lange, R. E. W. Hancock, J. Samuel, and W. H. Finlay. *In vitro* aerosol delivery and regional airway surface liquid concentration of a liposomal cationic peptide. *J. Pharm. Sci.* 90:1647-1657 (2001).
 - 26. T. R. Desai, J. P. Wong, R. E. W. Hancock, and W. H. Finlay. A novel approach to the pulmonary delivery of liposomes in dry powder form to eliminate the deleterious effects of milling. *J. Pharm. Sci.* 91:482-491 (2002).
 - 27. S. B. Kulkarni, G. V. Betagiri, and M. Singh. Factors affecting microencapsulation of drug in liposomes. *J. Microencapsul.* 12:229-246 (1995).
 - 28. A. Omri, M. Ravaoarinoro, and M. Poisson. Incorporation, release and *in-vitro* antibacterial activity of liposomal aminoglycosides against *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* 36:631-639 (1995).
 - 29. P. Diot, P. F. Dequin, B. Rivoire, F. Gagnadoux, F. Faurisson, E. Diot, E. Boissinot, A. Le Pape, L. Palmer, and E. Lemarie. Aerosols and anti-infectious agents. *J. Aerosol Med.* 14:55-64 (2001).
 - 30. S. L. Katz, S. L. Ho, and A. L. Coates. Nebulizer choice for inhaled colistin treatment in cystic fibrosis. *Chest* 119:250-255 (2001).